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RESEARCH PAPERS - 10TH SPECIAL ISSUE ON GRAPEVINE TRUNK DISEASES

Detection and quantification of black foot and crown and root rot pathogens in grapevine nursery soils in the Western Cape of South Africa

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Summary. Black foot disease (BFD) and crown and root rot (CRR) are important soilborne diseases that affect young grapevines in nurseries and vineyards. A 3-year survey (2013–2015) of five open-field grapevine nurseries was conducted in the Western Cape Province of South Africa. The survey involved the isolation of BFD and CRR pathogens from grafted rootstocks (ten plants per nursery, per year) that were rooted in soil for 1 year. In 2013 and 2015, grapevines were sampled, while in 2014, sampling was focused on rotation crops and weeds (ten plants each). The rotation crops included white mustard, lupins, canola, triticale and forage radish. The weed species sampled included Johnson grass, ryegrass, winter grass, Cape marigold and corn spurry. Soil samples from ten sites per nursery were also collected in close proximity to the sampled plants, at depths of 0–30 cm and 30–60 cm (ten samples per depth). Isolations were made from the grapevines, rotation crops and weeds. Pathogen detection and quantification in the soil were determined using quantitative real-time polymerase chain reaction technology. The predominant BFD pathogens isolated from grapevines were *Campylocarpon fasciculare*, *Ca. pseudofasciculare* and *Dactylonectria macrodidyma*. The predominant CRR pathogens were *Pythium irregulare* and *Phytopythium vexans*. *Dactylonectria macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *Py. irregulare*, *Py. ultimum* var. *ultimum* and *Py. heterothallicum* were isolated from triticale roots. *Dactylonectria* spp. were also isolated from corn spurry, while *Py. irregulare* and *Py. ultimum* var. *ultimum* were isolated from numerous weeds and rotation crops. Mean soil DNA concentrations of *Ilyonectria* and *Dactylonectria* were from 0.04 to 37.14 pg μL^{-1}, and for *Py. irregulare* were between 0.01 and 3.77 pg μL^{-1}. The *Phytophthora* mean soil DNA concentrations ranged from 0.01 to 33.48 pg μL^{-1}. The qPCR protocols successfully detected and quantified BFD and CRR pathogens in grapevine nursery soil. This is the first report of *D. pauciseptata* and *D. alcacerensis* in South African grapevine nurseries.

Keywords: *Dactylonectria*, *Pythium*, weeds, rotation crop, qPCR.

Introduction

The South African grapevine industry comprises 120,000 ha, approx. 100,000 ha wine grapes and 20,000 ha table grapes (SAWIS, 2017). In 2013, the wine industry contributed more than R36 billion to the annual GDP of South Africa, which amounts to 1.2% of total GDP. The wine industry supports 289,151 employment opportunities (Anonymous, 2015). South Africa produced 10.8 million hectolitres of wine in 2017, ranking as the 7th largest wine producer in the world (OIV, 2018). The South African table grape industry exported 62 million 4.5 kg cartons during 2017–2018, making it the 7th largest exporter, which is 6.5% of the world's export value (Lombard, 2018). Both of these industries are of crucial importance to the South African economy.

Black foot disease (BFD) and crown and root rot (CRR) are important diseases that affect young grapevines, in nurseries and vineyards. Both of the diseases contribute to the eventual decline of grapevines (Spies *et al.*, 2011; Agustí-Brisach *et al.*, 2013a).

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Symptoms of BFD include brown discolouration and streaking of vine vascular tissue, gum inclusions of the xylem vessels, reduced grapevine vigour, and sunken necrotic root lesions. This leads to shortened plant internodes and reduced root biomass. Death in young vines occurs rapidly whereas decline and death in mature vines occur at slower rates (Grasso and Magnano, 1975; Scheck *et al*., 1998; Gubler *et al*., 2004; Halleen *et al*., 2006). The outward symptoms of BFD are often indistinguishable from other grapevine trunk diseases, such as Petri disease. Crown and root rot symptoms in grapevines often manifest as brown/black discoloured rot of roots, stunted growth, chlorosis and wilting. Die-back and decline may also be observed. Stem cankers may also be caused by *Phytophthora* spp. (Chiarrappa, 1959, Marais, 1979, Zentmyer, 1980, Latorre *et al*., 1997, Fourie and Halleen, 2001).

Black foot disease is a complex, caused by species from the five genera *Ilyonectria* P. Chaverri and C. Salgado, *Dactylonectria* L. Lombard and Crous, *Campylocarpon* Halleen, Schroers and Crous, *Cylindrocladiella* Boesew., *Neonectria* Wollenw. and *Thelonectria* P. Chaverri and C. Salgado (Halleen *et al*., 2004; 2006; Cabral *et al*., 2012c; Carlucci *et al*., 2017; Scheck *et al*., 1998). The following BFD pathogens have been isolated from grapevines in South Africa: *I. liriodendri* (Halleen, Rego and Crous) P. Chaverri and C. Salgado, *D. macrodidyma* (Halleen, Schroers and Crous) L. Lombard and Crous, *D. novozelandica* (A. Cabral and Crous) L. Lombard and Crous, *D. torresensis* (A. Cabral, Rego and Crous) L. Lombard and Crous, *Ca. fasciculare* Schroers, Halleen and Crous and *Ca. pseudofasciculare* Halleen, Schroers and Crous (Halleen *et al*., 2004; 2006; Cabral *et al*., 2012c; Carlucci *et al*., 2017). In Spain, *Cylindrocladiella parva* (P.J. Anderson) Boesew. and *Cy. peruviana* (Bat., J.L. Bezerra and M.P. Herrera) Boesew. have been found to be associated with BFD (Agustí-Brisach *et al*., 2012). In South Africa, these *Cylindrocladiella* species were isolated from grapevines (Van Coller *et al*., 2005), but their role as BFD pathogens has yet to be established.

Crown and root rot of grapevines is caused by species in three oomycete genera, *Pythium* Pringsh., *Phytopythium* Abad, de Cock, Bala, Robideau, A.M. Lodhi and Lévesque and *Phytophthora* de Bary. The *Pythium* spp. include *Pythium aphanidermatum* (Edson) Fitzp., *Py. heterothallicum* W.A. Campb. and F.F. Hendrix, *Py. irregulare* Buisman, *Py. rostratum* E.J. Butler, *Py. sylvaticum* W.A. Campb. and F.F. Hendrix *and Py. ultimum* Trow (Marais 1979; 1980; Gubler *et al.* 2004; Spies *et al*., 2011). The *Phytophthora* species identified as causal agents were *Phytophthora cactorum* (Lebert and Cohn) J. Schröt., *Ph. cambivora* (Petri) Buisman, *Ph. cinnamomi* Rands, *Ph. cryptogea* Pethybr. and Laff., *Ph. megasperma* Drechsler, *Ph. niederhauserii* Z.G. Abad and J.A. Abad and *Ph. nicotianae* Breda de Haan. *Phytopythium vexans* (de Bary) Abad, de Cock, Bala, Robideau, Lodhi and Lévesque is the only *Phytopythium* species known to be a causal agent of CRR (Marais 1979; 1980; Gubler *et al.* 2004; Spies *et al*., 2011). All of the above oomycete pathogens, except *Ph. cambivora*, have been found to cause CRR in South Africa.

BFD and CRR are soilborne diseases for which little is known of pathogen levels in grapevine nursery soils. Quantitative real-time PCR (qPCR) is a sensitive, rapid and high-throughput method of detection and quantification of micro-organisms (Hardegger *et al*., 2000). Protocols have been developed for qPCR detection of CRR pathogens present in grapevine roots in South Africa (Spies *et al*., 2011). The qPCR detection of the '*Cylindrocarpon*' genus (= *Ilyonectria* and *Dactylonectria* spp.) in grapevine nursery soils was conducted in Spain by Agustí-Brisach *et al*. (2014), who found BFD pathogens in most of the soils sampled from nurseries and rootstock mother fields in Spain. Determination of presence and amounts of BF and CRR pathogens in grapevine nursery soils in South Africa needs to be determined, and this would aid in development of disease control strategies.

Management options for BFD in South Africa is limited to the use of cultural practices, as no registered fungicides are available (Van Zyl, 2011) and host resistance is not known. Practices that can be employed include limiting the predisposing stress factors such as improper planting holes and soil compaction (Larignon, 1999; Fourie *et al*., 2000), and hot water treatment of nursery grapevines (Halleen *et al*., 2007; Gramaje *et al*. 2011). Several studies were conducted to test fungicides against BFD pathogens (Rego *et al*., 2005; Halleen *et al*., 2007; Alaniz *et al*., 2011). Although some showed some promise, further trials are needed to confirm field efficacy. Studies on host resistance to BFD are limited (Gubler *et al*., 2004; Jaspers *et al*., 2007; Alaniz *et al*., 2010). Gubler *et al*. (2004) found that rootstocks *Vitis riparia* O39-16 and Freedom had some degree of resistance to *'C.' destructans*, while Alaniz *et al*. (2010) found the rootstock 110-R to be the most susceptible to *'C'. lriodendri* and 'C'. *macrodidymum*.

Several studies have examined management of crown and root rot on grapevines (Williams and Hewitt, 1948; Von Broembsen and Marais, 1978; Marais and Hattingh, 1986; Marais, 1988; Utkhede, 1992; Stephens *et al.*, 1999; Gubler *et al.*, 2004). Cultural practices such as preventing soil compaction and water logging conditions have been suggested (Utkhede, 1992; Gubler *et al.*, 2004). Von Broembsen and Marais (1978) also showed that hot water treatment (50°C for 30 min) reduced *Ph. cinnamomi* propagules in grapevine rootstocks. In South Africa, the fungicide fosetyl-Al is registered for use against soilborne diseases of grapevines (Van Zyl, 2011). Soil fumigation using methyl bromide, metam sodium or dazomet has also been shown to reduce *Phytophthora* and *Pythium* populations in grapevine nursery soils (Marais and Hattingh, 1986; Stephens *et al.*, 1999). The use of methyl bromide has recently been phased out in South Africa (UNEP, 2017). A study by Marais (1988) determined that the rootstock 143B Mgt had the greatest tolerance to *Phytophthora* while the rootstocks 99-Richter and 110-Richter were very susceptible.

The planting of cover crops in perennial cropping systems and nurseries is common practice. These are planted for various reasons including; prevention of soil erosion by winter rain (Baumgartner *et al.*, 2005), soil temperature regulation (Fourie and Freitag, 2010), weed suppression (Mohler, 2001; Blaser *et al.*, 2006), facilitation of nitrogen fixation (Parkin *et al.*, 2006), carbon sequestration (Reicosky and Forcella, 1998), and because some cover crops possess fungicidal, bactericidal, nematicidal and/or insecticidal properties (Brown and Morra, 1997; Kruger *et al.*, 2013). Many different cover crops are planted including legumes, C_3 and C_4 grasses or brassicaceous crops (Vukicevich *et al.*, 2016). Gamliel and Stapleton (1993) demonstrated that soil amended with cabbage residues had negative effects on *Py. ultimum*. Mattner *et al.* (2008) showed that isothiocyanates released during biofumigation suppressed '*C*'. *destructans*, *Py. ultimum* and *Ph. cactorum*. Berlanas *et al.* (2018) showed that biofumigation with white mustard reduced inoculum of *D. torresensis* and the incidence and severity of black foot of grapevine. Two studies have also shown the beneficial effect of mustard meal on reducing BFD inoculum in soil (Bleach *et al.*, 2010; Barbour *et al.*, 2014).

Grapevine nursery surveys have been conducted for the detection of black foot pathogens (Alaniz *et al.*, 2007; Dubrovsky and Fabritius, 2007; Petit *et al.*, 2011; Agustí-Brisach *et al.*, 2013). In South Africa, the most recent nursery surveys for BFD were conducted by Halleen *et al.* (2003), and for CRR by Spies *et al.* (2011). Soil has been confirmed as a major inoculum source for BFD pathogens (Agustí-Brisach *et al.*, 2013b; 2014). However, the presence and amounts of BFD and CRR pathogens have not been investigated in South African grapevine nursery soils. The objectives of the present study were: i), to quantify BFD and CRR pathogens in five grapevine nursery soils in the Western Cape over a 3-year period; and ii), to assess the levels of infection by these pathogens in grapevines, rotation crops and weeds growing in close proximity to the soil sampling locations.

Materials and methods

Plant and soil sampling

In 2013, 2014 and 2015, plant material and soil samples were collected from five open field nurseries located in the Western Cape of South Africa. Due to the crop rotation systems used in these nurseries, rooted grapevine plants were sampled in 2013 and 2015, while in 2014, rotation crops and weeds were sampled. Sampling of these plants were carried out in W-shaped patterns across the fields. Each year ten vines were sampled per nursery, one plant per site. No distinction was made between the cultivars sampled, so various rootstocks were sampled. All the sampled grapevines were visually healthy. Approximately three rotation crop plants and/or weeds were sampled at each site. The sampled rotation crops include Canola (nursery A, C), white mustard (nurseries A, B, C), forage radish (nursery C), triticale (nursery D) and lupins (nursery E). The weeds sampled included Johnson grass, ryegrass, winter grass, Cape marigold and corn spurry. The sampling was carried out at approximately the same sites every year. Nursery E employs a 3-year crop rotation system and as a result, rotation crops and weeds were sampled in 2014 and 2015. Additionally, soil samples were taken at the site where the plants were collected. Soil samples were taken with a soil auger approx. 10 cm from the grapevine plants, at two depths (0–30 cm and 30–60 cm) and placed in separate bags. The soil samples were then placed at -20°C until processing. A subsample of each soil sample was sent for soil analyses to Bemlab (Strand, South Africa), and for particle analyses to the Central Analytical Facilities at Stellenbosch Univer-

sity. Soil wetness classes were used to determine the soil wetness index of Lambrechts *et al.* (1978).

Isolations from grapevine rootstocks, rotation crops and weeds

Isolations were made from grapevine roots and basal ends for CRR and BFD pathogens. After thoroughly rinsing the roots under running tap water, ten randomly selected root pieces per plant were plated onto PARP medium (Jeffers and Martin, 1986) amended with Switch® fungicide (cyprodonil 375 g kg^{-1} plus fludioxonil 250 g kg^{-1}; Syngenta) for isolation of *Pythium* and *Phytopythium* spp., and ten randomly selected root pieces per plant onto PARPH medium for the isolation of *Phytophthora* species. The roots were then surface sterilised in 70% ethanol for 1 min. and left to air dry. Isolations were then made for BFD pathogens from the roots and basal ends (five pieces of root and five pieces of basal end tissue per plant) (Figure 1) onto two Petri dishes containing potato dextrose agar (PDA, Biolab, Randburg) amended with streptomycin (0.04 g L^{-1}; PDAS). For the rotation crop plants and weeds, isolations were only made from roots (ten randomly selected root pieces per plant) onto PDAS. The Petri dishes were incubated at 25°C for 1 week and any growth was transferred onto fresh PDA plates.

The weed species were identified using the guides by Henderson and Musil (1987); Stirton, (1987); Henderson (1995); Bromilow (2001) and Henderson (2001).

DNA extraction from mycelia and grapevine soil

DNA was extracted from mycelia of all fungal and oomycete isolates using a modified CTAB DNA extraction protocol based on that of Lee *et al.* (1990). The modifications were as follows: 1), harvested mycelia was macerated using 0.5 g of glass beads which were shaken at 30 Hz in a Retsch MM301 mixer/miller (Retsch, GmbH and Co.) for 5 min. ; and 2), two chloroform-isoamylalcohol steps were performed instead of one, to enhance the purification of the DNA. DNA concentrations were determined using a NanoDrop UV spectrophotometer (NanoDrop Technologies).

The soil samples were removed from the freezer and allowed to thaw after which the soil was thoroughly mixed and left to air-dry in sterile Petri dishes for 2 d. The dried soil aggregates were each crushed using a sterile spatula. Soil DNA extractions were carried out using the NucleoSpin Soil kit (Macherey-Nagel GmbH and Co.), according to the manufacturer's instructions. The SL1 lysis buffer was used together with the enhancer SX. The DNA was extracted from 0.5 g of soil per sample. Two DNA extractions were carried out on each soil sample (2 × 0.5 g) at each depth (0–30 cm or 30–60 cm) resulting in four DNA extractions per site (ten sites per nursery). The DNA was eluted in 100 µL of buffer SE. All soil DNA samples were diluted five times in sterile deionised PCR grade water before use in qPCR.

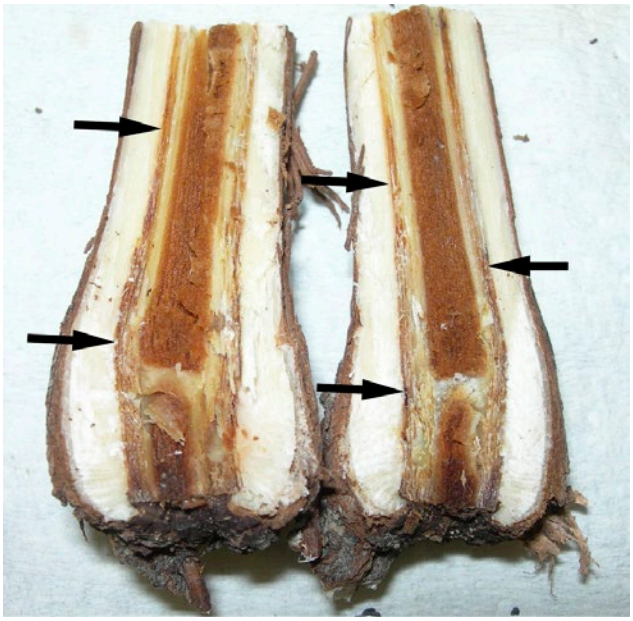

Figure 1. The sites in a grapevine rootstock base from which black foot disease pathogens were isolated (black arrows). Brown discolouration and streaking can be observed in the xylem tissues.

Species identification

Campylocarpon, Dactylonectria and Ilyonectria identification

Specific primers. DNA of suspected BFD pathogens was diluted to 25 ng μL^{-1} and was subjected to species-specific PCR using the following primer pairs (on the beta-tublin region); CymaF1 and CymaR2 to screen for the *Dactylonectria macrodidyma* complex, CyliF1 and CyliR1 for *Ilyonectria liriodendri*, CafaF1 and CafaR1 for *Campylocarpon fasciculare*, and CapsF1 and CapsR1 for *Ca. pseudofasciculare* (Mostert *et al.*, 2010) (Supplementary data, Table 1). The PCR reactions were set up separately for each primer pair using 1 × NH_4 buffer

(Bioline USA Inc.), 1.5 mM $MgCl_2$ (Bioline), 0.2 mg bovine serum albumin (BSA) Fraction V (Roche Diagnostics), 0.2 mM of each dNTP, 0.4 mM of each primer and 0.5 U of BIOTAQ (Bioline). The PCR reaction was conducted in an Applied Biosystems 2720 thermal cycler (Applied Biosystems) using a touchdown cycling programme, with an initial denaturation temperature of 94°C for 5 min, then 94°C for 45 s with five cycles at 66°C for 30 s, five cycles at 62°C for 30 s and 20 cycles at 60°C for 30 s, with an extension step at 72°C for 60 s and a final extension step at 72°C for 6 min. The PCR products were resolved on a 1.5% agarose gel (Lonza) stained with ethidium bromide and viewed on a UV transilluminator (Syngene).

Sequencing to identify isolates belonging to the *D. macrodidyma* complex and confirmation of *I. liriodendri* specific products. DNA samples that were identified as *D. macrodidyma* complex were further subjected to sequencing of the histone H3 (HIS) gene region to resolve the individual species in the *D. macrodidyma* species complex. The HIS gene was also amplified for selected isolates that were positive with the *I. liriodendri* specific primers. The primers CYLH3F and CYLH3R were used to amplify 500 bp of the partial HIS gene, according to Crous *et al.* (2004). The PCR consisted of 1 × NH_4 buffer (Bioline), 1 mM $MgCl_2$ (Bioline), 0.2 mg bovine serum albumin (BSA) Fraction V (Roche), 0.2 mM of each dNTP, 0.25 μM of each primer and 0.5 U of BIOTAQ (Bioline). The PCR product was run on a gel as previously described, purified using the MSB Spin PCRapace kit (STRATEC Molecular GmbH), and sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. All the samples were sequenced on an ABI 3130XL Genetic Analyzer by the Central Analytical Facilities at Stellenbosch University.

Sequencing of negative samples and isolates positive with *Campylocarpon* specific primers. PCR of the ITS region was conducted on all the remaining samples that could not be identified using the species-specific PCR approaches. A subset of the samples that were positive with the *Campylocarpon* specific primers was also selected for ITS PCR. ITS was chosen for *Campylocarpon* species confirmation because this gene region is adequate to distinguish these species. For the fungal samples, the universal fungal primers ITS1 and ITS4 were used (White *et al.*, 1990) to amplify 550 bp of ITS regions. The reactions consisted of 1 × NH_4 buffer (Bioline), 2 or 2.5 mM $MgCl_2$ (Bioline), respectively, for fungi or oomycetes, 0.2 mg bovine serum albumin (BSA) Fraction V (Roche), 0.2 mM of each dNTP, 0.2 μM of each primer, 0.5 U of BIOTAQ (Bioline) and 50 ng of target DNA. The PCR was conducted in an Applied Biosystems 2720 thermal cycler with an initial denaturation temperature of 95°C for 3 min followed by 35 cycles each at 95°C for 1 min, 50°C for 1 min, 72°C for 90 s, and a final extension step at 72°C for 5 min. PCR products were purified and sequenced as described above.

Oomycete identification

Oomycete cultures were identified by sequencing the ITS region. The oomycete PCR was carried out using the primer pair ITS4 (White *et al.*, 1990) and ITS6 (Cooke and Duncan, 1997). The same protocol was used as described above for the fungal ITS amplification and sequencing, and PCR products were purified and sequenced also as described above.

Phylogenetic analyses

The resulting HIS and ITS sequences were edited and aligned, and consensus sequences were generated, using Geneious R10.1.3 (Biomatters Ltd) (Kearse *et al.*, 2012). The consensus sequences were then compared to sequences in GenBank using the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences used in the phylogenetic analyses were lodged in GenBank. Reference sequences for each taxonomic group were obtained from GenBank and aligned with representative sequences from this study, from two isolates where possible, using the MAFFT V7.222 program with the L-INS-I method (Katoh *et al.*, 2002) in Geneious R10.1.3. Maximum likelihood (ML) analyses were carried out using PHyML (Guindon and Gascuel, 2003) under the general time reversible (GTR) model. The gamma distribution and proportion of invariable sites were assessed. One hundred replicates were used to calculate the bootstrap support values, and the clades with bootstrap values of equal to or greater than 70% were considered to be significant and highly supported (Hillis and Bull, 1993).

Quantitative real-time polymerase chain reaction

The total soil DNA was used for DNA quantification of black foot and root and crown rot pathogens

using the qPCR protocols described by Tewoldemedhin *et al.* (2011) for *Dactylonectria* and *Ilyonectria* spp., and Spies *et al.* (2011) for *Phytophthora* and *Py. irregulare*. Five-fold DNA dilution series were made from DNA of reference cultures of *D. macrodidyma* (SL281), *Phytophthora cinnamomi* (STE-U 7392) and *Py. irregulare* (STE-U 6752). DNA concentrations of 0.8 ng μL^{-1}, 0.16 ng μL^{-1}, 0.032 ng μL^{-1}, 0.0064 ng μL^{-1}, 1.28 pg μL^{-1}, 0.256 pg μL^{-1}, 51.2 fg μL^{-1}, 10.2 fg μL^{-1} and 2.04 fg μL^{-1} were used to set up a standard curve for DNA quantification. The concentration standards were carried out in triplicate and the soil DNA samples were carried out in duplicate.

The qPCR assay for the detection of *Ilyonectria* and *Dactylonectria* spp. consisted of the following; 1 × KAPA SYBR FAST qPCR master mix (contains SYBR Green I and $MgCl_2$ at 2.5 mM), (KAPA Biosystems) 0.3 μM of each genus specific primers YT2F and Cyl-R, and 2 μL of five times diluted DNA. The $MgCl_2$ concentration was adjusted to 4.5 mM by the addition of extra $MgCl_2$ (Bioline USA Inc.), and the final reaction volume was adjusted to 20 μL using sterile deionised PCR grade water (Bioline). The no template controls received 2 μL of sterile deionised water instead of DNA. The qPCR was carried out at an initial denaturation temperature of 95°C for 10 min, and 60 cycles each at 95°C for 10 s, 60°C for 10 s and 72°C for 30 s. In addition, melt curve analysis was included in the run at temperatures between 65 to 95°C with 1.0°C increments at 5 s intervals.

Phytophthora species in the soil samples were detected and quantified using the primers published by Schena *et al.* (2006), as optimised for use with SYBR Green I by Spies *et al.* (2011). This protocol uses a genus-specific primer pair Yph1F and Yph2R. Each qPCR reaction consisted of 1 × KAPA SYBR FAST qPCR master mix (with 2.5 mM $MgCl_2$), 0.3 mM of each primer Yph1F and Yph2R, and 2 μL of five times diluted DNA. Each reaction was adjusted to 20 μL using sterile deionised PCR grade water (Bioline). No template controls were included in each run. The qPCR cycling conditions consisted of an initial denaturation at 95°C for 10 min, then 50 cycles each at 95°C for 10 s, 62°C for 15 s and 72°C for 30 s. Melt curve analysis was included in each run at temperatures between 65 to 95°C with 1.0°C increments at 5 s intervals.

The protocol used for the detection and quantification of the root rot pathogen *Py. irregulare* was developed by Spies *et al.* (2011). Each reaction consisted of 1 × KAPA SYBR FAST qPCR master mix (contains SYBR Green I and $MgCl_2$ at 2.5 mM) (KAPA Biosystems), 0.3 μM of primer PirF1 and 0.9 μM of PirR3, and 2 μL of five times diluted DNA. The $MgCl_2$ concentration was adjusted to 3 mM by the addition of extra $MgCl_2$ (Bioline), and the final reaction volume was adjusted to 20 μL using sterile deionised PCR grade water (Bioline). No template controls and concentration standards were included in each run. The cycling conditions for each run consisted of an initial denaturation of 95°C for 10 min, then 50 cycles each at 95°C for 10 s, 65°C for 5 s and 72°C for 20 s. Melt curve analysis was included in each run, as described above.

Selected DNA concentration standards were included in triplicate in each run to enable DNA quantification after importing a saved standard curve. The qPCR analyses were carried out on a RotorGene 6000 real-time rotary analyser (Qiagen Inc.).

Subsets of the *Phytophthora* (ten samples) and *Py. irregulare* (ten samples) qPCR products were sequenced using the sequencing reaction protocol described above, with the same primers that were used in the qPCR reaction. No products of the qPCR assays for *Ilyonectria* and *Dactylonectria* spp. were sequenced since these species were abundantly isolated in comparison with only one retrieved isolate of *Phytophthora*.

qPCR inhibition testing

Nursery soil was sterilised by autoclaving (121°C and 103.4 kPa for 20 min), three times, on three consecutive days. Soil DNA extractions were carried out on the sterile soil using the Nucleospin soil kit (Macherey-Nagel GmbH and Co.). Three dilutions; 10×, 100× and 1,000×, were made of each extracted DNA sample and one sample was left undiluted. Ten ng of *I. liriodendri* DNA was added to each dilution and the undiluted samples. These DNA extractions were then tested using the qPCR assay described above. DNA samples without added *I. liriodendri* DNA were also tested for the presence of *I. liriodendri*. The quantitation cycle (C_q) values were recorded and subjected to analysis of variance using SAS (V9.3, SAS Institute Inc.).

Statistical analyses

The experimental design was a completely randomised design with ten replicates (sites) per nurs-

ery. The treatment design was a combined split plot design. Five nurseries were studied and combined after homogeneity of nursery variances were verified (Brown and Forsythe, 1974). Means for two technical and biological repeats were calculated for each soil sampling depth and site. The data were transformed using the natural logarithm function prior to analyses. The data were then subjected to analysis of variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.2; SAS Institute Inc.). Observations over time (year) were combined in a split-plot analysis of variance with year as sub-plot factor (Cramer *et al*., 1989). Shapiro-Wilk tests were performed on the standardized residuals from the model to verify normality (Shapiro and Wilk, 1965). Fisher's least significant difference (LSD) was calculated (at $P = 0.05$) to compare treatment means (Ott and Longnecker, 2001). A probability level 0.05 was considered statistically significant for all significance tests.

Results

Soil sampling and analyses

The soil analyses revealed that the texture of the soil sampled from nurseries A, B and D were coarse sand, from nursery C was a loamy coarse, and from nursery E was a loamy medium sand. For nurseries A, B, C and D there were no differences in soil texture between the sampling at 0-30 cm and 30–60 cm, but for nursery E the soil was a loamy medium sand at depth 0–30cm and loamy coarse sand at 30–60cm (Table 1). Of the different soil elements tested, only boron was low in nurseries B and D. The resistances measured of the soil at 30 to 60 cm depth for nurseries A and D were low, indicating excess of salts (resistance levels of below 300 Ohm are seen as problematic). Soils at the nurseries A, B, C and D were prone to wetness and their soil wetness indices ranged from 3 to 6, in contrast with nursery E which had a wetness index of less than 3.

Pathogen isolation and species identification

Brown discolouration and streaking was often observed in the xylem tissues of the nursery vines (Figure 1). Successful amplification was obtained by using the species-specific PCR for BFD pathogens. The identities of *D. alcacerensis*, *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *D. torresensis* and *I. liriodendri* were confirmed with phylogenetic analyses of the histone gene region (Supplementary data, Figure 1). All the species isolates grouped with reference sequences of the respective species, with bootstrap support of 74% or greater. *Campylocarpon pseudofasciculare* and *Ca. fasciculare* was confirmed with an ITS-rDNA phylogeny grouping with bootstrap support of 97% or greater with the reference sequences (Supplementary data, Figure 2). The CRR species were confirmed with phylogenetic analyses of the ITS-rDNA sequences (Supplementary data, Figures 3 to 5). *Phytophthora niederhauserii* was the only species within this genus, and this grouped with the reference sequence with 90% bootstrap support (Supplementary data, Figure 3). *Phytopythium helicoides*, *Pp. litorale* and *Pp. vexans* were identified and grouped with bootstrap support of 90% or greater with reference sequences of these species (Supplementary data, Figure 4). Five species of *Pythium* were identified. *Pythium irregulare*, *Py. rostratum*, *Py. sylvaticum* and *Py. ultimum var. ultimum* grouped with reference sequences of these species with bootstrap support of 98% or greater (Supplementary data, Figure 5). Two isolates grouped with *Pythium heterothallicum* and *Py. glomeratum* with 100% bootstrap support. These two species form an unresolved species-complex.

A total of 176 black foot pathogens was isolated. In 2013, 86 BFD isolates were obtained, 18 were obtained in 2014 and 72 in 2015. The incidence of the BFD pathogens over all the vines were: 20% for *D. macrodidyma*, 12% for *Ca pseudofasciculare*, 9% for *Ca. fasciculare*, 6% for *D. novozelandica*, 4% for *D. pauciseptata*, 2% for *D. torresensis* and 1% for both *D. alcacerensis* and *I. liriodendri*. In 2013, 19 CRR isolates were obtained, 19 in 2014, and 70 in 2015. The total of CRR pathogen isolates was 108. Overall vine incidence CRR pathogens was 18% for *Py. irregulare*, 11% for *Pp. vexans*, 9% for *Py. heterothallicum*/*Py. glomeratum*, 4% for *Py. ultimum var. ultimum*, 2% for *Pp. helicoides* and 1% each of *Pp. litorale*, *Py. rostratum*, *Py. sylvaticum* and *Ph. niederhauserii*.

Grapevine infections

In 2013, the black foot pathogens were the predominant species isolated from roots and basal ends of grapevine rootstocks based on the number of infected plants. Among these, *D. macrodidyma* was the predominant species, isolated from 22 plants in four

Table 1. Selected characteristics of the soil samples taken at 0–30 cm and 30–60 cm depths from five grapevine nurseries (A–E).

Nursery	Soil texture	Depth (cm)	pH	Stones (%)	Carbon (%)	Resistance (Ohm)	P Bray II (mg kg^{-1})	K (mg kg^{-1})	Cu (mg kg^{-1})	Zn (mg kg^{-1})	Mn (mg kg^{-1})	B (mg kg^{-1})	Fe (mg kg^{-1})	Clay (%)
A	Coarse sand	0-30	6.9	18	1	1050	808	114	9.56	37.5	42.0	0.41	188.61	6.18
A	Coarse sand	30-60	7.1	18	0.84	280	53	241	7.76	33.7	38.1	0.44	178.04	6.07
B	Coarse sand	0-30	6	6	0.66	3300	207	52	14.61	15.5	19.3	0.12	92.67	6.31
B	Coarse sand	30-60	6.1	6	0.66	1370	196	80	18.55	19.1	23.3	0.14	97.22	6.83
C	Loamy coarse sand	0-30	6.3	9	0.6	2610	188	54	3.34	40.6	16.4	0.25	269.73	10.46
C	Loamy coarse sand	30-60	6.2	11	0.74	1920	135	48	4.53	34.1	22.0	0.50	875.51	10.61
D	Coarse sand	0-30	6	11	0.74	620	117	56	4.53	21.4	25.9	0.20	76.47	5.43
D	Coarse sand	30-60	5.8	13	0.71	390	133	55	5.86	25.1	33.2	0.14	93.67	5.04
E	Loamy medium sand	0-30	6.6	1	0.22	1340	56	89	1.70	1.4	7.8	0.43	121.07	8.29
E	Loamy coarse sand	30-60	6.6	1	0.17	1340	44	78	1.31	1.1	5.7	0.65	88.69	8.04

out of five nurseries (Table 2). This was followed by *Ca. fasciculare* which was isolated from six plants in three out of five nurseries. *Phytopythium vexans* was the predominant oomycete infecting five plants in two nurseries. Nursery B and C each had the most infected plants (seven plants), with plants from nursery B containing the only *Phytophthora* species. No pathogens were obtained from nursery E. Fungal isolates were first stored at 4°C and thereafter plated again for identification. Due to this process, several of the putative pathogen isolates did not grow again after storage and could not be identified. The total number of black foot isolates could therefore have been more from the 2013 isolations.

In 2015, *Ca. pseudofasciculare* was the predominant fungal pathogen isolated from 20 plants (out of 40 plants) across four nurseries. This was followed by *Ca. fasciculare* which infected 11 plants from four nurseries. The predominant oomycete pathogen was *Py. irregulare* (20 infected plants) followed by *Pp. vexans* (15 infected plants). Nursery C had the greatest number of infected plants (10 plants) followed by nursery B (seven plants). Nursery A had the least infected plants (three plants).

Table 2. The number of plants infected by black foot and crown and root rot pathogens in five nurseries sampled in 2013, 2014 and 2015.

Disease	Pathogen	Number of infected plants[a]														
		2013 Nursery					2014[b] Nursery					2015 Nursery				
		A	B	C	D	E	A	B	C	D	E	A	B	C	D	E[c]
Crown and root rot	*Pythium irregulare*	-	1	-	-	-	-	-	4	4	4	3	2	3	2	10
	Pythium ultimum var. *ultimum*	-	-	-	-	-	-	-	2	1	-	-	2	-	1	1
	Pythium heterothallicum	-	-	-	-	-	-	-	-	2	-	-	2	10	1	1
	Pythium rostratum	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pythium sylvaticum	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-
	Phytopythium helicoides	-	1	2	-	-	-	-	-	-	-	-	-	-	-	-
	Phytopythium litorale	-	1	-	-	-	-	-	-	-	-	-	-	-	1	-
	Phytopythium vexans	-	3	2	-	-	-	-	-	-	-	2	4	6	1	2
	Phytophthora niederhauserii	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Black foot	*Dactylonectria alcacerensis*	-	-	1	-	-	-	-	-	-	-	1	-	-	-	-
	Dactylonectria macrodidyma	5	7	7	3	-	-	1	-	3	-	1	5	2	3	-
	Dactylonectria novozelandica	-	1	4	-	-	-	-	-	2	-	1	1	-	2	-
	Dactylonectria pauciseptata	-	3	-	-	-	-	-	-	2	-	-	2	-	1	-
	Dactylonectria torresensis	1	1	-	-	-	-	-	-	-	-	-	-	1	-	-
	Ilyonectria liriodendri	-	-	-	1	-	-	-	-	-	-	-	1	-	-	-
	Campylocarpon fasciculare	-	3	2	1	-	-	-	-	-	-	2	6	2	1	-
	Campylocarpon pseudofasciculare	-	-	3	-	-	-	-	-	-	-	1	7	8	4	-

[a] At least ten plants were sampled per field per year (approx. 50 plants per year).
[b] Rotation crops and weeds sampled in 2014. The rotation crops for nurseries were: A, Canola, white mustard; B, white mustard; C, Canola, white mustard, forage radish; D, triticale and E, lupins.
[c] Nursery E uses a 3-year crop rotation system, so no grapevines were planted in 2015, and only weeds and rotation crops were sampled in that year.

Five *Dactylonectria* species, *I. liriodendri*, *Ca. fasciculare* and *Ca. pseudofasciculare* were recovered from nursery grapevines during this survey in 2013 and 2015. The *Dactylonectria* species included *D. macrodidyma*, *D. novozelandica*, *D. torresensis*, *D. alcacerensis*, and *D. pauciseptata*. The CRR pathogens that were isolated from grapevines included five *Pythium* spp., three *Phytopythium* spp. and one *Phytophthora* sp. These species were *Pythium irregulare*, *Py. sylvaticum*, *Py. ultimum* var. *ultimum*, *Py. heterothallicum*, *Py. rostratum*, *Pp. helicoides*, *Pp. litorale*, *Pp. vexans*, and *Ph. niederhauserii.*

Isolations from rotation crop plants and weeds

In 2014, rotation crop plants and weeds were sampled from the five nurseries (Table 3). In nursery A, no pathogens were isolated from either rotation crop plants or weeds. In nursery B, no pathogens were isolated from the rotation crop plants; however, one isolate of *D. macrodidyma* was obtained from corn spurry. In nursery C, one isolate of *Py. irregulare* was obtained from forage radish, while one isolate of *Py. irregulare* was obtained from winter grass and two isolates of *Py. irregulare* were obtained from ryegrass. Two *Py. ultimum* var. *ultimum* isolates were also obtained from ryegrass and Cape marigold. Nursery D had the greatest diversity of pathogens isolated from the triticale plants, while no pathogens were isolated from the weeds. Four isolates of *Py. irregulare* as well as two of *Py. ultimum* var. *ultimum* were obtained from triticale roots. In nursery E, four *Py. irregulare* isolates were obtained from Johnson grass and three unknown weed species. No pathogens were isolated from lupin plants. In 2015, weeds were sampled from nursery E due to its 3-year rotation system. Ten weed plants were infected with *Py. irregulare*, and one plant each with *Py. ultimum* var. *ultimum* and *Py. heterothallicum*/*Py. glomeratum*. In addition, two plants were infected with *Pp. vexans*.

qPCR inhibition testing

Quantitative PCR amplification was successful and amplified the added *I. liriodendri* DNA with an efficiency of 88.4%, and the standard curve had a correlation coefficient of 0.993 to the concentration standards. The C_q values were recorded for each sample at the various soil DNA dilutions. The C_q values across all dilutions were similar, indicating no to very little qPCR inhibition.

Quantitative real-time polymerase chain reaction for pathogens from soil

Ilyonectria and *Dactylonectria, Py. irregulare* and *Phytophthora* spp. DNA were detected and quantified in soil samples.

The fluorescence obtained during the SYBR Green I assays for *Ilyonectria* and *Dactylonectria* species reached 100%. The efficiencies for the standard curves ranged between 80 to 86%, with R^2 values of 0.99. The minimum and maximum amounts of *Ilyonectria* and *Dactylonectria* DNA detected across all nurseries were 0.04 pg μL^{-1} and 37.14 pg μL^{-1}. The melting temperatures ranged between 85 to 87°C. The DNA melting temperature for the standard DNA (*D. macrodidyma*) was 86.5°C.

The fluorescence obtained during the *Phytophthora* assay reached 100% with standard curve reaction efficiencies between 80 and 100% with R^2 values of 0.99. The melting temperatures for the *Phytophthora* species ranged between 82 to 88°C. The DNA melting temperature for the standard DNA (*Ph. cinnamomi*) was 85°C. The minimum and maximum amounts of *Phytophthora* DNA detected in a nursery was 0.01 pg μL^{-1} and 29.53 pg μL^{-1}.

The assay for the detection of *Py. irregulare* reached a fluorescence of 100% with standard curve reaction efficiencies between 78 and 100% with R^2 values of 0.99. The melting temperature for the *Py. irregulare* amplicons was 79.5°C. The DNA melting temperature for the standard DNA (*Py. irregulare)* was 79.5°C. The minimum and maximum amounts of DNA detected in a nursery were 0.01 pg μL^{-1} and 3.77 pg μL^{-1}.

The qPCR results of *Dactylonectria* and *Ilyonectria* spp. showed that these species were present in all the samples tested, except for one site in nursery A in 2013. In only one sample (nursery A, site 6, 2015) at the sampling depth of 30-60 cm, were *Dactylonectria* and *Ilyonectria* spp. not detected. There were no significant differences between the two soil sampling depths ($P = 0.206$), so quantities from both depths were combined (Table 4). Statistically significant differences for the interaction between nursery and year were detected ($P < 0.001$). In 2015, nursery A had a significantly greater DNA concentration than all the other nurseries and years. Following this, nurseries B, C and D in 2015, and nurseries A and C in 2014 had concentrations that did not differ significantly from each other. Nursery E had the lowest DNA concentration which did not significantly differ from nurseries B, C and D in 2013, and nurseries B and D in 2014.

Table 3. Pathogens isolated from weeds and rotation crop plants sampled from four grapevine nurseries in 2014.

Nursery[a]	Site[b]	Common name	Genus and species	Family	Weed or rotation crop	Pathogen
B	9	Corn spurry	*Spergula arvensis*	Caryophyllaceae	Weed	*D. macrodidyma*
C	1	Ryegrass	*Lolium temulentum*	Poaceae	Weed	*Py. irregulare*
		Winter grass	*Poa annua*	Poaceae	Weed	*Py. irregulare*
	2	Forage radish	*Raphanus sativus*	Brassicaceae	Rotation Crop	*Py. irregulare*
		Ryegrass	*Lolium temulentum*	Poaceae	Weed	*Py. irregulare*
	4	Cape marigold	*Arctotheca calendula*	Asteraceae	Weed	*Py. ultimum* var. *ultimum*
		Ryegrass	*Lolium temulentum*	Poaceae	Weed	*Py. ultimum* var. *ultimum*
D	1	Triticale	*x Triticosecale*		Rotation crop	*D. pauciseptata*
	4	Triticale	*x Triticosecale*		Rotation crop	*Py. irregulare*
	5	Triticale	*x Triticosecale*		Rotation crop	*Py. irregulare* *D. pauciseptata*
		Triticale	*x Triticosecale*		Rotation crop	*Py. ultimum* var. *ultimum* *Py. heterothallicum/ glomeratum* *Py. irregulare* *D. novozelandica*
	8	Triticale	*x Triticosecale*		Rotation crop	*D. macrodidyma* *D. novozelandica*
	9	Triticale	*x Triticosecale*		Rotation crop	*D. macrodidyma*
	10	Triticale	*x Triticosecale*		Rotation crop	*Py. irregulare* *Py. heterothallicum/ glomeratum* *D. macrodidyma*
E	3	Johnson grass	*Sorghum halepense*	Poaceae	Weed	*Py. irregulare*
		Unknown weed				*Py. irregulare*
	6	Unknown weed			Weed	*Py. irregulare*
	8	Unknown weed			Weed	*Py. irregulare*

[a] No BFD or CRR pathogens were recovered from weeds and rotation crop plants sampled from nursery A.
[b] Ten sites were sampled per nursery field.

Pythium irregulare was detected in most of the samples tested, except from six sites in nursery A, nine sites in nursery B and four sites in nursery D in 2013. Furthermore, in 2014, *Py. irregulare* was not detected in one site each in nurseries B, C and D. In 2015, no detections were made from one site in nursery A. There were no significant differences between the two soil sampling depths ($P = 0.397$) and quantities for both depths were therefore combined (Table 4). Statistically significant differences for the interaction between nursery and year were detected ($P < 0.001$). Greater concentrations were observed in 2015 for nurseries A, B, C and D, but the mean concentration of nursery C in this year was not significantly differ-

Table 4. Mean *Dactylonectria* and *Ilyonectria*, *Pythium irregulare* and *Phytophthora* DNA concentrations in soil, determined with quantitative real-time PCR analyses of soils from five grapevine nurseries sampled over 3 years.

Nursery	Year	Mean soil DNA concentration (pg µL^{-1})[a]		
		Dactylonectria* and *Ilyonectria	***Pythium irregulare***	***Phytophthora***
A	2013	2.10[b] (1.13[c] d)	0.07[b] (0.07[c]ef)	0.11[b] (0.10[c] f)
	2014	4.82 (1.76 bc)	0.04 (0.04 f)	1.45 (0.90 de)
	2015	10.14 (2.41 a)	0.83 (0.61 a)	10.47 (2.44 a)
B	2013	0.33 (0.29 ef)	0.01 (0.005 f)	0.17 (0.15 f)
	2014	0.49 (0.40 ef)	0.10 (0.09 ef)	2.45 (1.24 c)
	2015	6.04 (1.95 bc)	0.50 (0.41 bc)	4.96 (1.78 b)
C	2013	0.80 (0.59 e)	0.21 (0.19 de)	0.13 (0.12 f)
	2014	4.17 (1.64 c)	0.02 (0.02 f)	2.14 (1.14 cd)
	2015	6.01 (1.95 bc)	0.39 (0.33 cd)	7.74 (2.17 a)
D	2013	0.33 (0.29 ef)	0.08 (0.08 ef)	0.16 (0.15 f)
	2014	0.55 (0.44 ef)	0.04 (0.04 f)	0.92 (0.65 e)
	2015	6.38 (2.00 b)	0.64 (0.50 ab)	2.97 (1.38 c)
E	2013	0.30 (0.26 f)	0.08 (0.08 ef)	0.04 (0.04 f)
	2014	0.25 (0.23 f)	0.06 (0.06 ef)	0.17 (0.16 f)
	2015	0.28 (0.24 f)	0.07 (0.07 ef)	2.24 (1.18 cd)
	LSD[d]	0.3137	0.1454	0.3129

[a] Log-transformed DNA concentrations in each column followed by the same letter do not differ significantly ($P > 0.05$), according to Fisher's LSD test
[b] Back transformed DNA concentrations
[c] Log transformed DNA concentrations
[d] Critical value for comparison according to the LSD test ($P = 0.05$)

ent from that obtained for this nursery in 2013. The remaining sampling of the nurseries in 2013 and 2014 had equally low DNA concentrations.

Phytophthora spp. were detected in most of the samples tested, except in three sites of nursery A in 2013. There were no significant differences between the two soil sampling depths ($P = 0.2516$), so these data were combined (Table 4). Significant differences for the interaction between nursery and year were observed ($P < 0.001$). In 2015, nurseries A and C had greater mean *Phytophthora* spp. concentrations, followed by nursery B. A middle group of nurseries D and E in 2015 and A, B, C and D in 2014 all had greater concentrations than all the nurseries in 2013, and D and E in 2014.

In general, greatest concentrations of pathogen DNA were measured from the 2015 sampling, except for the BFD pathogens and *Py. irregulare* in nursery E. Nursery E had the lowest DNA concentrations for all three pathogen types tested. Nursery A had greater DNA concentrations for *Ilyonectria* and *Dactylonectria* for all five nurseries over all three years, except for nursery C in 2014. For *Py. irregulare* nursery A only had greater DNA concentration in 2015 for nurseries B, C and E. *Phytophthora* DNA concentrations was greater for nursery A in 2014 in comparison with nurseries B and E, and in 2015 with nurseries B, D and E.

The sequences obtained from the *Phytophthora* qPCR product matched a published *Phytophthora* sp. sequence in GenBank. Similarly, the *Py. irregulare* sequences were very similar to published *Py. irregulare* sequences in GenBank.

Discussion

This was the first study to quantify DNA of black foot and root and crown rot pathogens in grapevine nursery soils in South Africa. The DNA of these pathogens was detected, using qPCR, from soil of all five nurseries in 2013, 2014 and 2015. Isolations from nursery grapevines confirmed the presence of these pathogens in the 2013 and 2015 samplings, from four of the five nurseries.

A wide diversity of black foot pathogens was identified in this study. These included five species of *Dactylonectria, Ilyonectria liriodendri, Campylocarpon fasciculare* and *Campylocarpon pseudofasciculare*. This is the first report of *Dactylonectria alcacerensis* and *D. pauciseptata* on grapevines in South Africa. The high frequency with which *Dactylonectria* species were isolated from nursery vines during this survey is in accordance with other studies conducted in Spain (Alaniz *et al*., 2007; Agustí-Brisach *et al*., 2014).

Of the root and crown rot pathogens, five *Pythium* spp., three *Phytopythium* spp. and one *Phytophthora* sp. were isolated. *Pythium irregulare*, *Py. sylvaticum*, *Py. ultimum* var. *ultimum*, *Py. heterothallicum*, *Py. rostratum*, *Pp. vexans*, and *Ph. niederhauserii* were isolated, and are known grapevine pathogens (Marais 1979, 1980; Spies *et al.* 2011). *Phytopythium helicoides* and *Py. litorale* have also been isolated from grapevines in nurseries and vineyards (this study; Spies *et al.* 2011), but their pathogenicity toward grapevine has not been determined. *Pythium sylvaticum* was isolated from nursery vines for the first time since 1980 (Marais, 1980). Only one *Phytophthora* isolate (*Ph. niederhauserii*) was obtained from nursery B in 2013. This is in accordance with Spies *et al.* (2011), who also only found *Ph. niederhauserii* in one nursery in the Wellington area of South Africa. The low occurrence of *Phytophthora* species in nursery vines can be attributed to the application of fosetyl-Al and metalaxyl based fungicides for the control of downy mildew. It is also known that *Phytophthora* spp. are difficult to isolate by direct plating as they tend to display weak growth in the presence of saprophytes (Erwin and Ribeiro, 1996), and this could also contribute to the low occurrence of this pathogen. Another reason for the difficulty in isolating *Phytophthora* from infected roots is due to the disintegration of the necrotic tissue during the rinsing and surface sterilisation processes (Bumbieris, 1972; Paulitz and Adams, 2003). Alternative approaches for the detection of *Phytophthora* in the soil can be attempted such as dilution plating and soil baiting with the appropriate baiting material (Erwin and Ribeiro, 1996). Dilution plating and soil baiting can also be used to test the viability of any propagules present in the soil. A direct approach to detection and quantification of pathogenic fungi and oomycetes is through the use of qPCR on root DNA samples, as in the studies by Spies *et al.* (2011) and Tewoldemedhin *et al.* (2011).

Of the different rotation crop plants investigated, only triticale (five plants) and forage radish (one plant) harboured black foot and crown and root rot pathogens. Canola, white mustard and lupins did not have any of these pathogens. Nurseries B and C, which were planted with Canola and white mustard, had greater numbers of grapevines infected with black foot and root and crown rot pathogens than other surveyed vineyards, indicating that these rotation crops had either little effect on soil-borne pathogens or that other factors contributed to the higher occurrence of pathogens in vines. It is known that lupins can harbour *Py. ultimum* (Weimar 1952; Simmons 1966), *Py. vexans* (Vanev *et al*. 1993) and several pathogenic *Phytophthora* spp. (Pennycook 1989; Rahman et al. 2014). From Canola, *Py. ultimum* (Pennycook 1989), *Py. irregulare* (Shivas 1989) and *Phytophthora* spp. (Erwin & Ribeiro 1996; Jung *et al*. 2011) have been reported. None of these pathogens have been found on white mustard (Farr *et al*. 2018). The potential for biofumigation with brassicaceous crops or products as a control strategy for management of black foot pathogens has been demonstrated (Bleach *et al.* 2010; Barbour *et al.* 2014; Whitelaw-Weckert *et al.* 2014). These plants release glucosinolates when incorporated into soil, which are then degraded into volatile isothiocyanates, and these compounds can suppress pathogenic fungi (Brown and Morra, 1997). However, studies have shown that biofumigant treatments may not affect some *Pythium* spp. (Stephens *et al*., 1999; Mazzola *et al*., 2001). Of the brassicaceous plants surveyed during the present investigation (Canola, forage radish and white mustard), only one forage radish plant tested positive for *Py. irregulare.* This plant was sampled from nursery C, which had loamy coarse sand with the greatest clay content of the nurseries surveyed. According to Brown and Morra (1997), clay and organic matter in soil may absorb the glucosinolates rendering them less effective against pathogens.

The weeds that harboured pathogens in the present study included corn spurry, Cape marigold and

the three grasses, ryegrass, winter grass and Johnson grass. A study in Spain by Agustí-Brisach *et al.* (2011) found that 26 weed species, including grasses (Poaceae) and flowering weeds in the Asteraceae, carried *Dactylonectria macrodidyma*. They also demonstrated that the *D. macrodidyma* isolated from weeds could induce typical black foot symptoms on grapevines. The present study showed that black foot pathogens and *Pythium* spp. can occur on weeds. Several *Pythium* spp. were also obtained from weed and grass samples in Japan (Uzuhashi *et al.*, 2010). French-Monar *et al.* (2006) showed that *Phytophthora capsici* was able to use Solanaceous weeds, in a vegetable field, as alternative hosts. Weeds can be sources of inoculum, but the relevance of the different weeds in terms of disease incidence needs to be determined before strategies can be recommended for weed removal. In addition, weeds may allow for the survival of pathogens through seasons when economic host crops are not grown (rotation or fallow periods).

The present study has demonstrated that qPCR is a sensitive, rapid and high-throughput method for detection and quantification of soil-borne pathogens. The established protocols of Spies *et al.* (2011) and Tewoldemedhin *et al.* (2011) used in this study were successfully adapted for the detection and quantification of pathogens in soil. In general, over the three years of sampling, there were increases in the mean DNA concentrations for all of the pathogens. The reasons for this are not clear. Halleen *et al.* (2003) suggested that there may be inoculum build-up of BFD pathogens during a 2-year rotation period. A period longer than 3 years is required to determine the extent inoculum build-up in soils. The presence of the pathogen DNA in the soil as well as in grapevine plants shows that pathogen inoculum persists in the soil during each crop rotation year. The DNA concentrations in the crop rotation year (2014) for *Dactylonectria* and *Ilyonectria* spp. and for *Phytophthora* spp., were equal or greater than in 2013, the first grapevine year investigated. Together with the fact that the DNA concentrations for these pathogens were greater in the second grapevine year investigated (2015), this indicates that the pathogens survive successfully in soil or in the roots and stem bases of specific weeds and rotation crop plants. Cardoso *et al*. (2013) and Berlanas *et al*. (2017) have demonstrated that BFD pathogens can survive in soil during rotation cycles in grapevine nurseries. *Dactylonectria*, *Ilyonectria* and *Phytophthora* spp. can all form chlamydospores, which are survival structures allowing these fungi to persist in soil (Erwin and Ribeiro, 1996; Halleen *et al*., 2004), while *Pythium* and *Phytophthora* spp. produce oospores that can also survive in soil (Van der Plaats-Niterink, 1981; Erwin and Ribeiro, 1996).

Nursery A generally had low numbers of infected plants, but high levels of black foot pathogen DNA were detected across all the years studied, and high levels of *Phytophthora* and *Pythium irregulare* DNA were found in 2015. Soil analyses from this nursery revealed greater amounts of phosphorous, potassium and manganese in this nursery compared to the other nurseries. It is possible that these levels of nutrients gave strong nursery plants, that were resistant to pathogen infection, despite the presence of pathogens in the soil.

The laboratory soil analyses showed nutrient amounts in all the soils that are considered to be within the normal ranges, except for boron in nurseries B and D, and low resistance levels for the deeper soil samplings for nurseries A and D (Dr F. Ellis, personal communication). Therefore, plant stress from nutrient deficiencies or salinity were deemed to be negligible in these nurseries. Berlanas *et al*. (2017) showed that there was a positive correlation between soil calcium carbonate and colony forming units of black foot pathogens. However, this compound was not analysed in the present study. The soil wetness index is an important characteristic which indicates the potential of a soil to become waterlogged. Four of the nurseries are situated in a production area with low-lying alluvial soils which are prone to wetness, and these nurseries also had greater soil wetness indices than nursery E, which has a sloping terrain and makes use of ridging to improve soil drainage. The soil type and the cultural practice of ridging probably contributed to lower pathogen amounts measured in the soil and absence of pathogens in the vines of this nursery. Other factors that could also contribute could be that nursery E is situated where there is not a long history of grapevine nursery cultivation, and a 3-year rotation system is applied in this nursery. Lupin is also used as a rotation crop, and these leguminous plants are known to produce saponins. These compounds are produced by many plant families as deterrents against pests and pathogens, and are released into the soil. Plant saponin composition can affect responses particular pests and pathogens (Moses *et al*., 2014). Deacon and Mitchell (1985) showed that saponins from oats could lyse *Pythium* and *Phytophthora* zoo-

spores. Although saponins may play a role, *Ilyonectria destructans*, *Py. irregulare*, *Py. ultimum*, *Py. vexans* and *Phytophthora cinnamomi* have been isolated from lupin species (Simmons 1966; Vanev *et al*. 1993; Mulenko *et al*. 2008; Bahramisharif *et al*. 2014). The effect of lupins and/or saponins on BFD and CRR pathogens would shed light on the potential of these plants for reducing diseases caused by these pathogens.

It is difficult to determine the biological relevance of the DNA concentrations determined with qPCR. The question remains as to how soil DNA concentrations will relate to disease severity. Various factors also influence these relationships, so the question cannot be answered only by pathogen presence in the soil.

The proliferation or suppression of soilborne plant pathogens and their abilities to infect and cause symptoms on their hosts is determined by complex combinations of biotic and abiotic components, and their interactions in soil environments. The present study has highlighted the possible effects of soil physico-chemical characteristics (e.g. nutrient status in nursery A, clay content in nursery C, differences in soil wetness indices), rotation crops (e.g. biofumigants), crop rotation systems (2- vs. 3-year rotation systems in nurseries A-D vs. E), and cropping histories (e.g. nursery E) on the incidence of BFD and CRR pathogens in grapevine nurseries in the Western Cape of South Africa. The complexity of interactions and diversity of factors involved preclude development of concrete conclusions and reliable recommendations for crop rotation systems to suppress soilborne pathogens and reduce disease in South African grapevine nurseries. Future research is required to focus on specific factors, reduce the impacts of external parameters, and monitor effects over a long periods (i.e. at least two full rotation cycles).

Nurseries are known to be sources of inoculum for black foot disease (Halleen *et al*. 2003; Agustí-Brisach *et al*., 2014) and root and crown rot pathogens (Spies *et al*., 2011). All the grapevines that were sampled appeared healthy, despite being infected with BFD and CRR pathogens. This confirms reports by Halleen *et al*. (2003) that symptomless plants harboured BFD pathogens. The present study also highlights the need for fungal pathogen testing before grapevines are certified pathogen-free. The results of the current study confirm the presence of decline pathogens in the soils of grapevine nurseries in South Africa. Triticale and forage radish harboured grapevine pathogens and would need to be considered in decision making over which rotation crop to plant in nurseries with a history of black foot and crown and root rot. Furthermore, a more extensive study over a longer period should be conducted on the suitability of different rotation crops currently used in grapevine nurseries in South Africa, and especially the contribution of *Brassica* crops to biofumigation. In the absence of registered fungicides against BFD pathogens, the only effective control method is hot water treatment of rooted grapevine plants (Halleen and Fourie, 2016). The fungicide fosetyl-Al is still the most effective means to control CRR pathogens, but hot water treatments of nursery stock may also be beneficial. Effective water drainage systems such as ridging and avoidance of soil compaction should also be implemented.

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